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13. ABSTRACT *(Maximum 200 Words)*

The major research focus of this grant is to develop effective treatments for patients with low volume metastatic disease using dendritic cell (DC)-based vaccines loaded with tumor RNA. Toward this goal, during the first year of this grant we have made the following progress: (1). We have shown that treatment of DC after transfection with RNA with TNF-α or CD40L enhances their function as measured by in vitro CTL induction. Based on these observations and pending additional optimizations we plan to incorporate the use of CD40L treatment in the clinical protocol. (2). We were able to isolate and amplify RNA from frozen tumor sections without apparent loss of function-as indicated by the ability of the amplified RNA to stimulate CTL responses. However, additional studies as outlined in the original grant applications will be necessary to optimize and adapt this strategy for clinical use. Successful RNA amplification may obviate the need of limiting patient accrual to those from whom sufficient cells can be generated (Specific aim #2) and proceed to the more encompassing protocol described in Specific aim #4. (3). We were unsuccessful in obtaining sufficient breast tumor cells for isolation-of sufficient quantities to preclude the need for amplification. We are developing an alternative approach using immunoselection columns in collaboration with Nexcell Therapeutics.

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TABLE OF CONTENTS

INTRODUCTION.

The major research focus of this application is to develop effective treatments for patients with low volume metastatic disease using autologous dendritic cell-based tumor vaccines. The proposed studies are based on our recent discovery that RNA pulsed DC of murine and human origin are highly effective stimulators of T cells and tumor immunity. A key advantage of using tumor RNA as source of antigen is that sufficient antigen can be generated by RNA amplification techniques from small tumor specimens. Ongoing phase I clinical studies with carcinoembryonic (CEA) peptide and CEA RNA transfected DC have demonstrated (so far) the safety of this treatment. Furthermore, preliminary analysis of patients treated with CEA RNA transfected DC show induction of CEA-specific T cell responses in the vaccinated patients. The central hypothesis of this proposal is that vaccination with tumor RNA transfected DC against a broad repertoire of tumor antigens expressed in patients with breast cancer will constitute an effective therapy for metastatic breast cancer.

Therefore, the primary objective of this research proposal is to develop optimal methods for using DC pulsed with tumor RNA as a broadly applicable treatment for patients with metastatic breast cancer. The specific objectives of the proposed study are:

1. To optimize antigen presentation by DC transfected with tumor RNA isolated from patients with metastatic breast cancer.

2. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with RNA isolated directly from tumor cells.

3. To develop methods to isolate, amplify, and enrich for biologically active mRNA from breast cancer tissue.

4. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with amplified tumor RNA.

BODY OF PROGRESS REPORT

According to the Statement of work the plans for Year 1 are:

27. Optimization of antigen presentation by RNA transfected DC- Effect of treatment with TNF-a and CD40L.

28. Develop methods for converting cellular mRNA into representative cDNA library.

29. Develop protocol for isolating breast tumor cells from bone marrow, isolate RNA and demonstrate induction of CEA CTL in vitro- in preparation of phase I clinical trial with directly isolated tumor RNA.

SOW-1: Optimization of antigen presentation by RNA transfected DC- Effect of treatment with TNF-a and CD40L.

Previous studies have suggested that RNA is taken up preferentially by immature DC **(35)**, consistent with the observation that immature but not mature DC exhibit extensive phagocytic and macropinocytic activity. It was, however, shown that immature DC generated in the presence of GM-CSF and IL-4 are unstable and revert to monocytes when cytokines are withdrawn **(ref)**. Moreover, mature DC are more potent APC than immature DC. We have therefore explored whether immature DC are more susceptible to RNA transfection and whether maturation of the transfected DC will enhanced their function, i.e. stimulation of CTL responses. We tested both TNF-α and CD40L previously shown to cause DC maturation in murine studies. As RNA we used CEA-specific RNA which represents a tumor associated antigen expressed in over 50% of breast cancer patients.

TNF-α. PBMC-generated DC were treated with TNF–α, before or after CEA RNA transfection. DC generated in the presence of GM-CSF and IL-4 express intermediate levels of MHC class II molecules and B7-1 (CD80) and are mostly $CD83^-$ (1). Following TNF–α treatment, PBMC-derived DC "mature", which correlates with the upregulation of class II and CD80 expression and cells become $CD83^+$ (2, 1). Maturation of DC is also accompanied by an increase in antigen presentation evidenced by enhanced MLR activity of mature versus immature DC (29).In the experiment shown in Figure 1, DC were generated in the presence of GM-CSF and IL-4, and the large, class II intermediate immature DC (approximately 50% of the total population) were further purified by cell sorting as described previously (1).

Figure 1A shows that treatment of sorted immature DC with TNF-α causes the upregulation of CD83 expression. Figure 1B shows that "immature" DC transfected with CEA RNA and then treated with TNF-α were significantly more potent stimulators of CEA-specific CTL than DC treated with TNF-α before transfection with RNA whereas treatment with TNF-α had no significant effect on the ability of CEA peptide-pulsed DC to stimulate a CEA-specific CTL response. This experiment has been performed twice but the conditions for TNF treatment have not yet been optimzed.

In other studies ongoing in our laboratory funded by other sources, TNF treatment of murine bone marrow-derived DC also induced phenotypic maturation of the DC as evidenced by

elevated expression of class II, CD80 and CD40. However, whereas the TNF treated DC were effective at presenting antigen in vitro, they were not able to stimulate CTL responses in vivo. Initial indication suggest that the TNF stimulated DC are prone to undergo apoptosis (unpublished data). We are therefore reluctant to introduce TNF treatment of DC into clinical settings as proposed in the grant application.

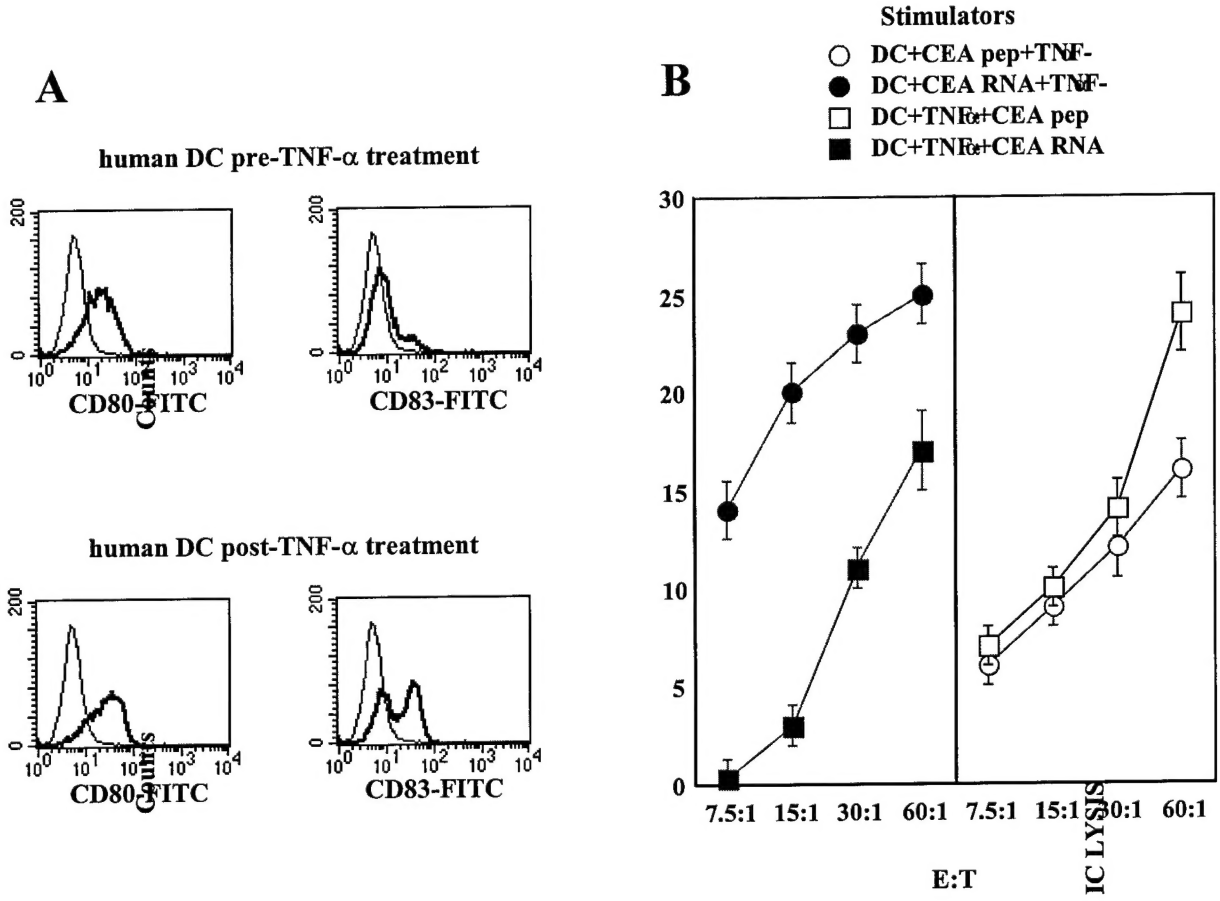

Figure 1. Effect of TNF–α treatment on the ability of CEA RNA-transfected DC to stimulate CTL *in vitro*. Panel A: DC were generated from PBMC as described (1) The large, class II intermediate cells were purified by cell sorting and analyzed pre- and post-treatment with TNF–α (100 ng/ml for 18 h) for the expression of CD80 (B7-1) and CD83. Panel B: Sorted DC were either incubated with CEA RNA or with CEA peptide followed by culture in the presence of TNF–α or cultured with TNF–α followed by RNA transfection or peptide pulsing. DC pulsed with CEA peptide and DC pulsed with HCV peptide were used as targets. Data represents the differences in the lysis of DC+CEA peptide and DC+HCV peptide.

CD40L. The effect of CD40L on RNA/transfected DC function was analyzed as described for TNF-α. CD40L was used in a soluble form as a trimer complex.CD40L is not commercially available and was obtained as a gift from Immunex corp. Figure 2 shows that treatment of DC with CD40L induces phenotypic maturation, as evidenced by the upregulation of several cell surface molecules, in particular CD80 (B7-1) and CD83.

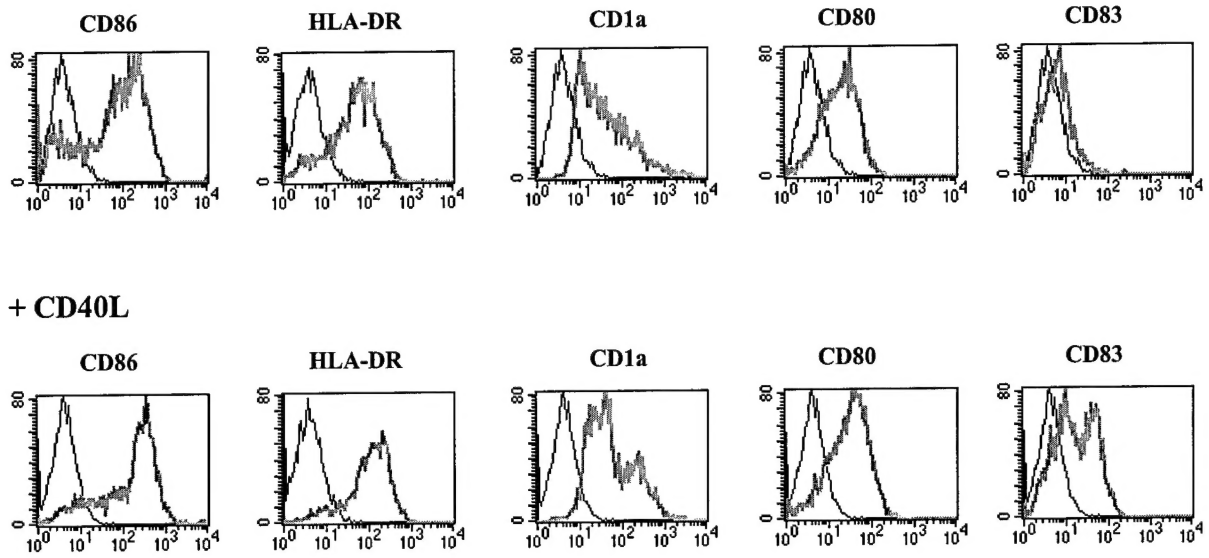

Figure 2. FACS analysis of DC before and after CD40L treatment. DC generated in GM-CSF and IL-4 for 7 days were harvested and a portion were cultured for an additional 18 h with CD40L 1 μg/ml. The DC were then stained with mAb to CD80, CD83, CD86, and HLA DR. CD83 was upregulated after CD40L exposure.

The functional consequences of CD40L treatment, i.e. stimulation of CTL responses following RNA transfection, was analyzed essentially as described for TNF-α shown in Figure 1B. The ability of DC loaded with antigen in the form of peptide to stimulate an antigen-specific CTL response was analyzed in Figure 3A. DC were pulsed with CEA peptide before or after treatment with CD40L. CD40L-matured DC were associated with greater CEA peptide-specific lytic activity if peptide pulsing was performed after maturation. Addition of the CD40L after loading peptide loading resulted in no improvement in CTL stimulation over that of DC which were not exposed to CD40L. The ability of DC loaded with antigen in the form of RNA to stimulate an antigen-specific CTL response was analyzed in Figure 3B. DC were transfected with CEA RNA pre- and post-treatment with CEA RNA. CD40L-matured DC were associated with greater CEA specific lytic activity if the CEA RNA transfection was performed before maturation. Addition of the CD40L before CEA RNA transfection resulted in similar CTL stimulation as that of DC which were not exposed to CD40L.

Thus, like in the case of TNF-α, RNA uptake was limited to immature DC. However, unlike the previous example, mature DC were more potent than immature DC in presenting peptide-antigen. The reason for this is not clear, a possible explantion is that in this instance maturation was accompanied by increases in MHC class I expression.

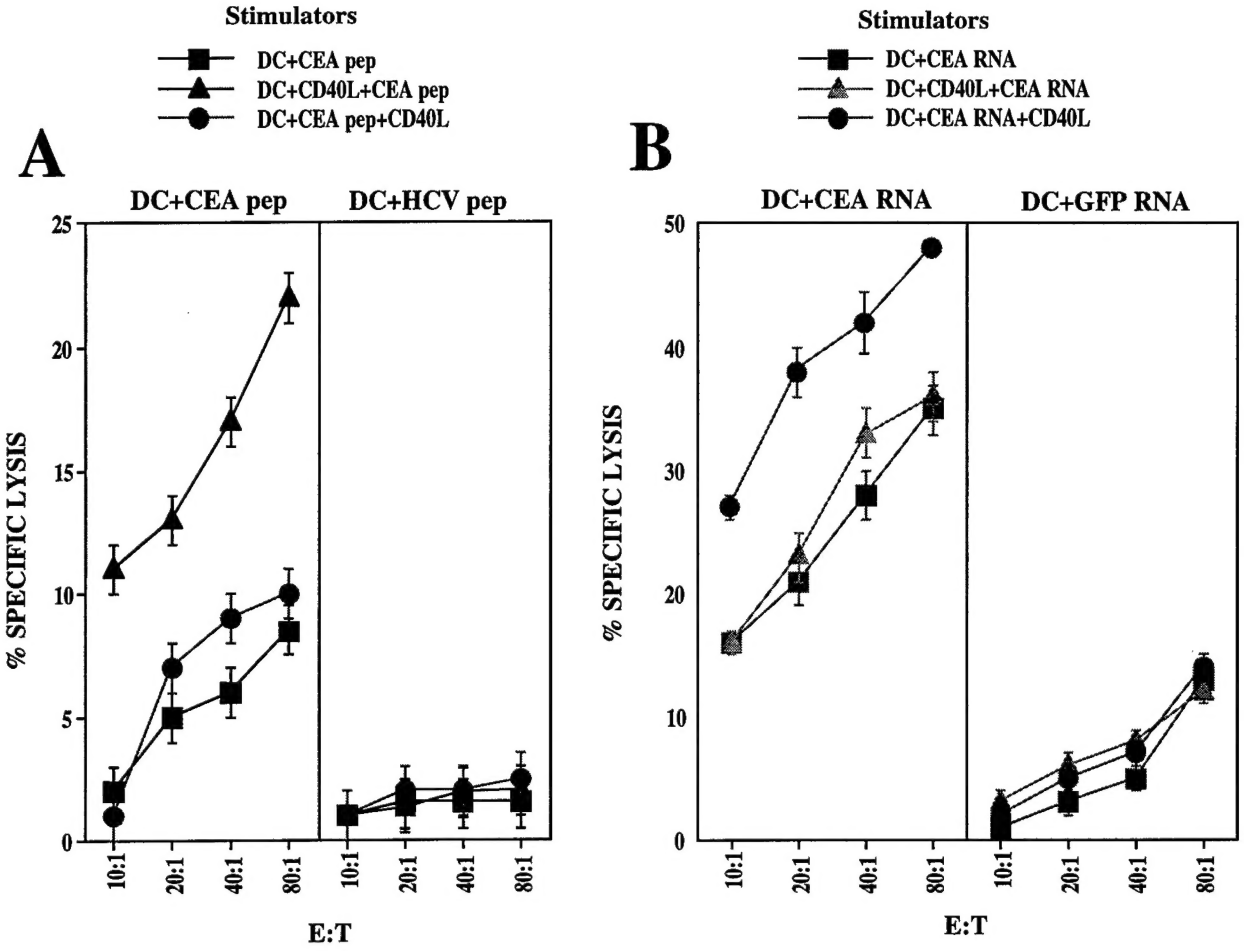

Figure 3. Stimulatory activity of DC pulsed with CEA peptide or transfected with CEA RNA pre- and post-CD40L exposure. Panel A: DC loaded with CEA peptide before and after 18 h incubation with CD40L were used as stimulators of autologous PBMC. Autologous DC loaded with CEA or HCV peptide were used as targets. Data is representative of three experiments. Panel B: DC transfected with 10 μg IVT CEA RNA before and after 18 h incubation with CD40L were used as stimulators of autologous PBMC. Autologous DC loaded with CEA or GFP RNA were used as targets.

Plans for next year:

Based on murine studies it appears that exposure of DC to TNF induces maturation but also predisposes the cells to apoptosis. This was not seen in the case of CD40L. Therefore our plans are as follows:

1. Further optimize CD40L treatment of DC-measuring CTL induction by RNA transfected DCA as a readout.

2. Incorporate CD40L treatment in the clinical protocol. The CD40L preparation that we are using was obtained from Immunex corp. Immunex has produced clinical grade CD40L and we have initiated discussion with the company to make it available for for clinical use.

SOW-2: Develop methods for converting cellular mRNA into representative cDNA library.

The source of tumor tissue used to generate cDNA libraries was CEA positive tumor biopsies provided as frozen sections.

Microdissection: We used initially the SURF procedure (3) described in the application but were generally unsuccessful in adapting this protocol to our samples. One reason may be that it was developed for paraffin-embedded tissue and we are using frozen section (in order to preserve the intactness of the RNA). We have therefore adopted a simpler, though more labour intensive two-step procedure involving microscope guided scraping of normal material sorrounding the tumor nodule followed by collection of the tumor material. Figure 4A shows an H&E stained frozen section from a CEA^+ colorectal liver metastasis showing a metastatic nodule surrounded by benign tissue. To isolate tumor cells, normal tissue surrounding the cancerous nodule is first removed (Figure 4B) and then the tumor cells are collected (Figure 4C). In this experiment, 35 nodules were isolated in a 3 hr procedure from two consecutive sections, pooled, and RNA was extracted. RNA yield was 2.24 μg, obtained from approximately 55,000 tumor cells. Gel analysis under denaturing conditions and ethidium bromide staining confirmed that the RNA was intact (data not shown).

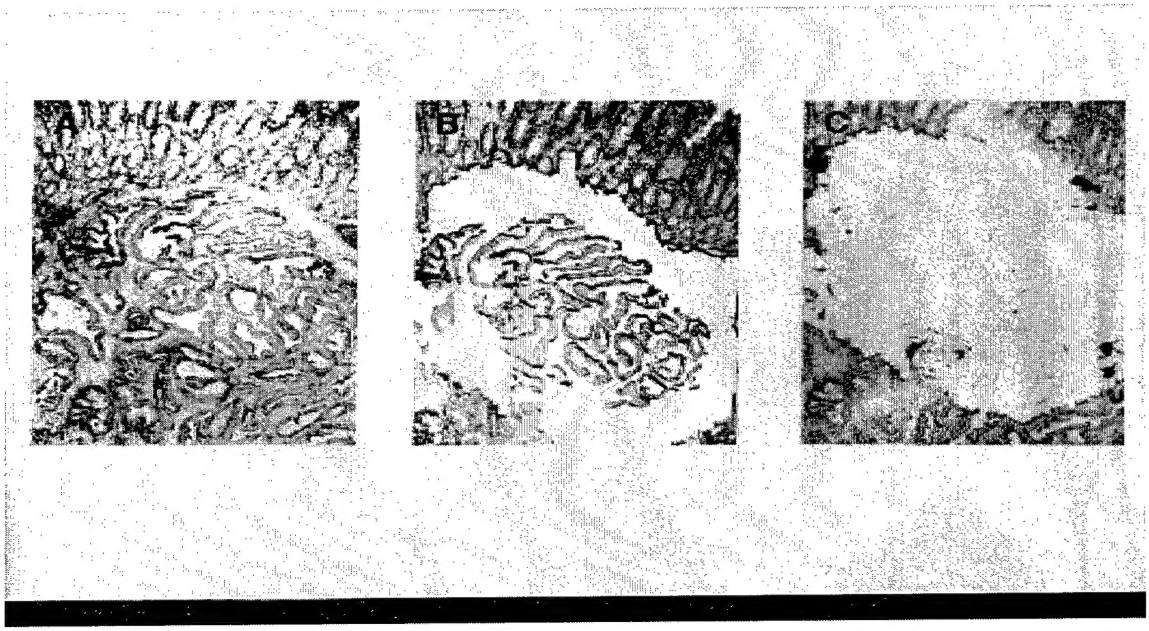

Figure 4: Microdissection of a colorectal metastatic nodule from a frozen section. A liver metastasis was resected for curative intent from a patient with CEA positive colorectal cancer. Frozen sections were cut at 20 nm in a cryostat mounted on glass slides and stained with H&E. Panel A shows a single tumor nodule sorrounded by normal tissue. Microdissection was performed using an inverted microscope and an attached mechanical micromanipulator for manipulating a glass capillary tube which is used as a cutting tool. Under 40X-400X magnification, tumor cells are separated from benign tissue by first removing the normal cells adjacent to the tumor nodule (B) and then collecting the tumor tissue in the middle (C). 35 tumor nodules were isolated and pooled from 2 slides and RNA extracted using phenol/chloroform and ethanol precipitation. RNA yield determined by UV absorption was 2.24 μg. The integrity of the RNA was confirmed by agarose gel electrophoresis under denaturing conditions and ethidium bromide staining (data not shown).

As pointed out, the procedure is labor intensive requires exquisite skills and not always reproducible. We have begun to explore the use of laser guided microdissection (LTM) protocols.

cDNA library generation: We are currently focusing on adapting and optimizing the SMART PCR cDNA Synthesis protocol from Clonotech combined with the RNase H⁻ MMLV RT from Gibco/BRL, as described in the original application. The cDNA preparations are used to transcribe RNA in vitro with T7 polymerase (the T7 promoter is encoded in the amplification primers) and the products are first analyzed on denaturing agarose gels, first by staining with ethidium bromide and subsequently blotted and hybridized to an actin probe. An example is shown in Figure 5.

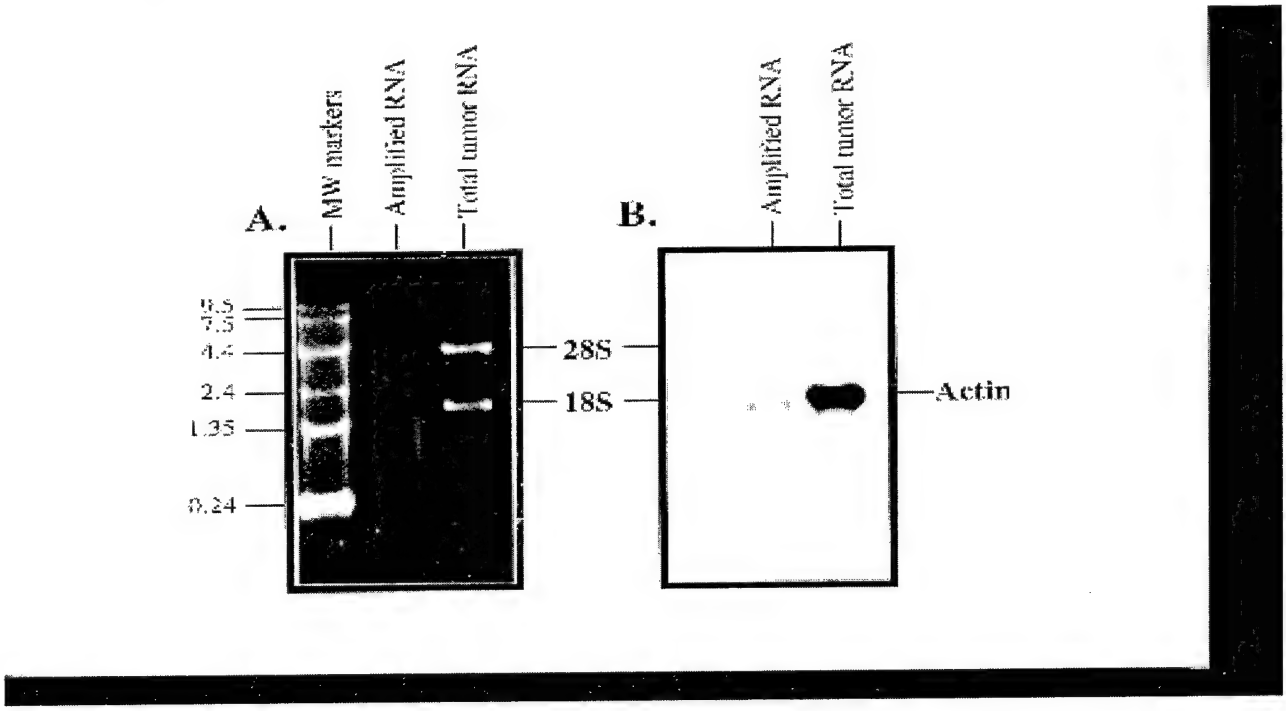

Figure 2: Biochemical analysis of RNA amplified from tumor cells. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturers protocol. 1μg of RNA was amplified using the Smart PCR synthesis protocol (Clonotech). Tumor RNA and the amplified RNA products were subjected to agarose/formaldehyde gel electrophoresis and stained with ethidium bromide (A). Alternatively, RNA was blotted and hybridized with an actin probe (B).

Staining with ethidium bromide (Figure 5A) shows that the predominant RNA species present before amplification correspond to the two ribosomal RNA species while the amplified RNA migrates as a heterogeneous population corresponding in size to the mRNA population in murine cells. To assess whether the amplified RNA species correspond to full length transcripts, the RNA displayed in Figure 5A was blotted and hybridized with an actin-specific random primed probe. In each case a single prominent band was seen which corresponds in size to the actin mRNA (Figure 5B).

Judging from the band intensities we estimate that the efficiency of generating full length actin during the amplification procedure was not more than 1-3%. These data show that the average size of the amplified RNA product corresponds to the expected size of the starting mRNA population, however, the efficiency of genrating full length RNA appears to be low; clearly there is room for improvement. We are currently testing various parameters of the amplification protocol. Next year we will start using alternative amplification strategies such as using oligo(dT) primers linked to paramagnetic beads for mRNA capture and "antisense RNA" techniques described in the original applications.

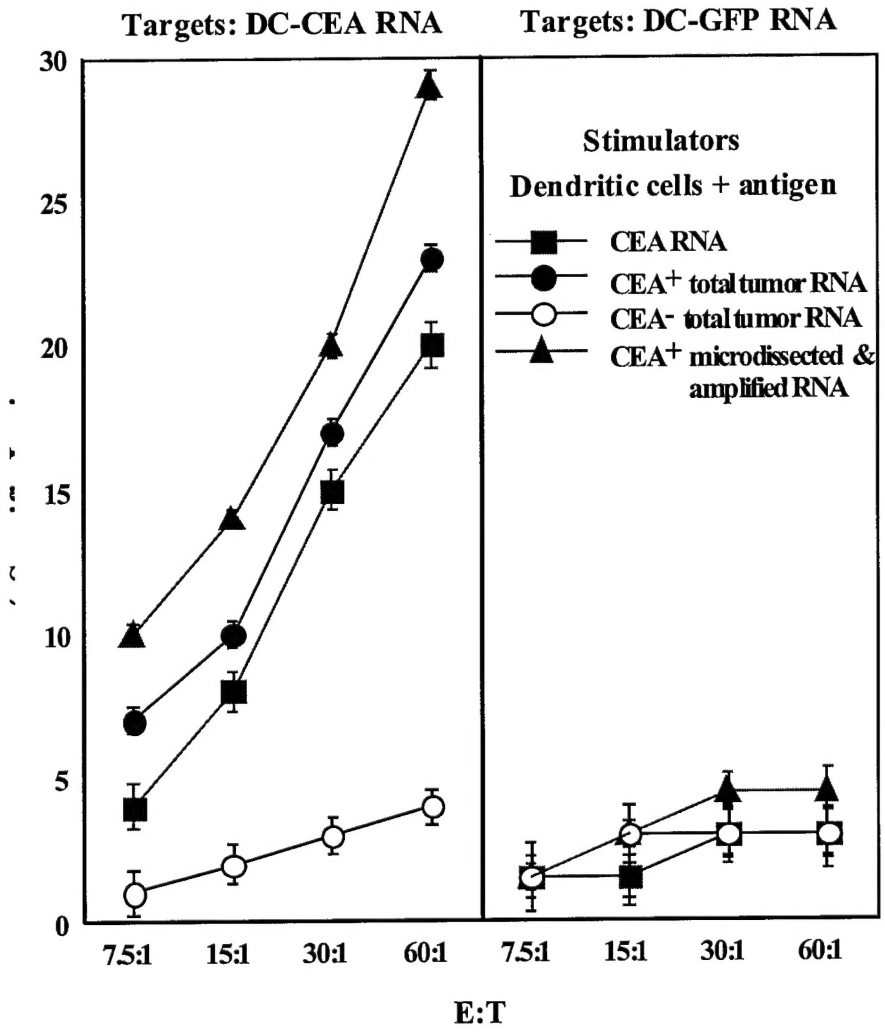

Figure 6: Induction of a primary, CEA-specific CTL response in vitro using DC transfected with RNA amplified from microdissected tumor cells. PBMC from a healthy volunteer were stimulated with autologous DC transfected with various RNA preparations and tested for the presence of CEA-specific CTL. DC transfected with in vitro synthesized CEA RNA or GFP RNA were used as specific and non specific targets, respectively, as previously. For stimulations, DC were transfected with in vitro transcribed CEA RNA, RNA

isolated from a CEA^+ cell line (SW1463), a CEA^- cell line(KLEB), and RNA amplified from the microdissected tumor cells described in Figure 4.

Functional analysis of amplified RNA: Despite the low efficiency of generating full length RNA we begun to evaluate the function of the amplified RNA, namely the ability to stimulate CTL responses in vitro following transfection into DC. The only experiment performed so far gave very encouraging results (Figure 6). Clearly, amplified RNA transfected DC stimulate a robust CTL response despite the fact that by gel analysis only a fraction of the mRNAs are full length and hence translatable.

Plans for the next year

1. Development of Laser Guided Microdissection (LTM) techniques. LTM equipment & training will become available.

2. Optimize RNA amplification protocols and apply optimized techniques to bone marrow derived breast tumor cells (see below).

SOW-: Develop protocol for isolating breast tumor cells from bone marrow, isolate RNA, and demonstrate induction of CEA CTL in vitro- in preparation for phase I clinical trial with directly isolated tumor RNA.

Over the past year, we have evaluated 35 patients (34 women, 31 Caucasion, 2 African American, and 1 male caucasion) with breast cancer for isolation of breast cancers cells for preparation of total tumor RNA.

Due to the use of stem cell growth factors, and the ability to harvest progenitor cells from mobilized blood, we have noted that bone marrow aspiration to harvest bone marrow for autologous bone marrow transplant is done much less frequently than previously. We attempted to utilize an alternative source of tumor cells, specifically, isolated tumor cells in other sites such as malignant ascites or malignant pleural effusions.

Twenty seven patients were not good candidates for isolation of purified breast cancer cells because they lacked pleural effusions or ascites which were accessible for drainage to obtain tumor cells. Five patients with metastatic breast cancer underwent therapeutic thoracentesis (three on two occasions for a total of 8 thoracenteses). All the thoracentesis specimens were processed with attempts to isolate sufficient quantities of tumor cells to generate total tumor RNA. In none of the cases were there enough tumor cells to isolate total tumor RNA. Therefore, no patient's breast cancer has been utilized to generate total tumor RNA that can be utilized in a clinical trial to date.

We have therefore begun to develop an alternative strategy for obtaining breast tumor cells using immumoisolation columns to isolate breast tumor cells from either bone marrow, or mobilized peripheral blood monuclear cells using antibodies and maganetic beads to "purge" the blood or bone marrow of tumor cells, generating a tumor cell preparation. This work is done in collaboration with investigators at Nexell Therapeutics, who have the antibodies, magentic beads, and columns necessary to "purge" bone marrow and mobilized peripheral blood monuclear cells. Pilot studies performed by collaborator Dr. Amy Ross at Nexell showed that the

tumor enrichment columns (TECs) were capable of recovering from 15% to 67% of tumour cells in PBMC and bone marrow samples, for an enrichment that is 20-2500 fold.

Plans for next year

In this second year of this study, we will optimize a protocol for the detection and isolation of breast cancer cells from archived PBMC and bone marrow samples of patients with known metastatic disease using tumour enrichment columns (TEC) provided by Nexell Therapeutics, Inc.

1. Confirm the utility of the Nexell protocol using PBMC "spiked" with CAMA-1 breast cancer cells.
2. Quantify tumor cell enrichment using the Miltenyi CD45 microbeads on a depletion column.
3. Determine whether serial tumour cell enrichment by the Nexell TEC followed by leukocyte depletion through Miltenyiís depletion column increases the purity of the isolated tumour cells without compromising the quantity of cells retrieved.
4. Use the protocols developed above to isolate metastatic breast cancer cells from samples of PBMC or bone marrow in patients with known metastatic disease from archived PBMC and bone marrow samples from patients with known metastatic(breast) disease.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of the beneficial effects of TNF–α and CD40L on DC function.
- Development of "1st generation" microdissection techniques yielding biologically active RNA
- Successful amplification of RNA from microdissected tissue without loss of immunological function (i.e. the ability to stimulate a CTL response in vitro following transfection of DC).

REPORTABLE OUTCOMES

N/A

CONCLUSIONS

1). TNF-a and CD40L treatment of RNA transfected DC improve CTL induction. CD40L treatment will be incorporated into the clinical program.

2). RNA can be isolated from microscopic amounts of tumor tissue-from fixed tissue on pathology slides- and amplified without loss of function. The microdissection techniques and amplification protocols will require extensive optimizations for clinical application.

3). We were unsuccessful in obtaining sufficient breast tumor cells for RNA isolation-of sufficient quantities which preclude the need for amplification. We are developing an alternative approach-using immunoisolation with TEC columns provided by and in collaboration with Nexcell Theapeutics.

If the amounts of tumor cells and RNA obtained by those procedures will be insufficient for use in a clinical protocol, we will incorporate an amplification step-as described in SOW-2- and will modify the planned clinical trial to include vaccination with amplified RNA.

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APPENDICES-NON